EXTRACTION AND QUANTIFICATION OF EXTERNAL MYCORRHIZAL HYPHAE
(For Soils Dominated by Silts and Clays or High in Particulate Organic Matter)
Detailed Version 5.1c

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Extraction Procedure

1. Prepare soil by carefully breaking it up and removing roots and rhizomes. Do not dry the soil. Discard large roots (>1 mm diameter) and rhizomes. Retain roots <1 mm in diameter (detach from larger roots when necessary). Push the moist soil through a 2-mm sieve (No. 10). Collect any roots (<1 mm diameter) that get caught in the sieve. Gather all roots <1 mm in diameter together and cut them into 1-2 mm segments over the sieved soil. Thoroughly mix soil and root segments. If samples were frozen, thaw them in a refrigerator overnight, and then bring them to room temperature before preparation. If prepared samples were frozen before hyphal extraction, thaw overnight, and mix well to evenly distribute any condensed moisture.

2. Determine dry wt/wet wt ratio of a 10-g subsample (for later conversion of data on an oven-dry basis).

3. Weigh two, replicate 5-g moist subsamples from each sample into two separate 600-mL beakers and record wet weights.

4. Add 250 mL d.i. water using volumetric flask and 31 mL sodium hexametaphosphate solution using 50-mL graduate cylinder to each beaker. Cover with parafilm or aluminum foil to prevent evaporation, and let soak overnight [Soaking overnight ensures more consistency between samples, and reduces the time required to break up aggregates. This may not be necessary for sandy soils or soils with poor aggregate stability.]

5. Stir sample with a glass stirring rod. Using a pipette, rinse stirring rod with 9 mL d.i. water into the beaker. Wipe stirring rod dry between samples.

6. Sonicate soil solutions for 20-25 seconds at 43 W (calibrated calorimetrically; North, 1976) while carefully rotating beaker. Using a pipette, rinse the tip of the sonicator with 10 mL d.i. water into the beaker, and touch the edge of the beaker to the tip to get the last drop of water into the beaker. [This makes a total volume of 300 mL added to the sample in the beaker.] Wipe sonicator tip dry between samples.

7. Place beaker on stir plate at high speed so that the solution vortex touches bottom of beaker and stir bar is visible. Reduce speed so that the bottom of the vortex is approximately halfway between the top of the spinning solution and the bottom of the beaker. [This will allow sand particles and any other large, heavy particles to settle to the bottom of the beaker while aliquots for dilution are being removed.]

8. Using a different wide-mouth pipette for each sample, dilute samples as follows. Fill pipette and eject to rinse sides of pipette with soil solution. Remove a 6-mL aliquot and transfer into another set of 600-mL beakers. For consistency, remove aliquots from halfway between the beaker edge and the vortex with the pipette tip immersed to the 9-mL mark. [Because of differential settling rates of materials in the soil suspension, quickly remove aliquot by drawing solution into the pipette to just above the desired mark. Quickly eject enough solution to line up the meniscus with the mark. If the mark is overshot or too much time is taken, do not alternately draw and eject solution until meniscus is positioned; rather, eject the entire aliquot back into the beaker and start over.] Rinse stir bar and wipe dry between samples. Flush pipettes with d.i. water and drain for reuse.

9. To each 6-mL aliquot, add 250 mL d.i. water and 31 mL sodium hexametaphosphate solution.

10. Stir diluted soil solution again at high speed to resuspend hyphae. Then reduce speed to the lowest setting that will keep the stir bar moving uniformly. Fill pipette and eject to rinse sides of pipette with soil solution. Take two 10-mL aliquots from each beaker using the same location and procedure as in Step 8 and transfer into a 50-mL long-cone centrifuge tube. Rinse and dry stir bar between samples.

11. Centrifuge at 1000 × g for 8 min (coast to stop).

12. Decant supernatant and discard.

13. Add 10 mL 50% glycerol to the remaining pellet and carefully vortex to resuspend.

14. Centrifuge resuspended pellet at 75 × g for 30 sec and coast to stop. (<u>Important:</u> it is necessary to time and stop this step using a clock rather than the timer on the centrifuge.) [Alternatively, if centrifuging brings too many hyphae into pellet, try letting suspensions settle on lab bench for a short but consistent period of time, e.g., 10 min.]

15. Immediately after the centrifuge stops (or the settling time is over) carefully decant the supernatants into filter holders with 20-µm nylon filters.

16. Turn vacuum on. After all supernatants have been pulled down, rinse each filter funnel twice.

17. Turn vacuum off, remove 20-µm nylon filter and slide it into a 15-mL centrifuge tube (with bottom side of filter against centrifuge tube wall). Add 5 mL of trypan blue stain to each tube, cap, and vortex for 30 sec, making sure filters come off tube wall and spin with stain solution. Stain for at least 1.5 hours.

18. Rinse funnel part of filter holders with d.i. water. After staining time is over, reset with white cellulose nitrate filters to collect hyphae. [Moisten sintered glass first to keep filter edges from wrinkling.]

19. Vortex each 15-mL tube for a few seconds and immediately decant into filter holders. [Make sure filters are still moist.]

20. Turn vacuum on. After all solutions have been pulled through, turn vacuum off. Fill each centrifuge tube with 8-10 mL d.i. water, cap, vortex, and pour into the respective funnels to rinse. Turn vacuum on until all rinse solutions are pulled through. Turn vacuum off and repeat this rinsing procedure until no blue color from the staining solution remains in the centrifuge tubes (usually only takes 2 times after first rinse). [Rinsing directly into the funnel with a wash bottle at this point causes the hyphae to clump for an uneven distribution which is difficult to count accurately. However, pouring 8-10 mL from the centrifuge tubes into the filter holders with the vacuum off appears to minimize this problem.]

21. With vacuum still on, remove the funnel part of the filter holder, and leave filters on the sintered glass with the vacuum on for about a minute to remove excess moisture from filters and help prevent filters from drying with wavy edges. Turn the vacuum off, remove filters, and place on labeled slides.

22. To further prevent filters from drying with wavy edges, dry filters on slides in a slide warmer or convective oven at 35°C (95°F) until dry. Large washers (1.25-1.5 inches in diameter) with holes that are as large as the filtered area on the membrane also can be used as weights while the membranes are drying to flatten them and to prevent wavy edges or curling. <u>Important:</u> filters must be thoroughly dry or they will not clear completely. It also appears that membranes clear better and are less likely to dry with wavy edges when they are relatively new (<1 year).

23. Mount filters on slides and clear them using low-viscosity, non-fluorescent immersion oil. Take one slide out of the slide warmer and while still warm, remove the dried filter from the slide, spread a spiral of oil the size of the filter, put the filter on, add another drop of oil on top, and top with a cover slip. [Ideally, use enough oil to fill the area under the cover slip but not so much that excess oil bleeds out across the slide from under the cover slip. It is possible to add more oil later if necessary. If the membranes are not completely flat, let slides sit overnight with large washers on top of cover slips.]

Notes

1. The first centrifugation (1000 × g) is intended to bring mycorrhizal hyphae down while leaving some of the finer colloids in suspension. The second centrifugation (75 × g) is intended to bring any large particles or organic pieces down while keeping the hyphae in suspension. Filtration through the 20-µm nylon filter is intended to catch hyphae while letting any remaining colloids pass through. The routine procedure follows the above steps in order and results in one white cellulose nitrate filter to count for each initial 5-g soil sample. However, to check the efficiency of the procedure for each soil type, we (1) filtered and stained the supernatant from the first centrifugation, (2) filtered and stained the supernatant from the 75 × g centrifugation (the filter obtained in the routine procedure), (3) repeated steps 13-21 with the pellet remaining after the 75 × g centrifugation of the routine procedure, and (4) continued to repeat steps 13-21 with the pellet remaining after each subsequent 75 × g centrifugation until no material was pelleted out [this usually takes a total of 2 pellet resuspensions after the one normally counted, but may take more in some soils]. By totaling the counts on all filters (usually 4), the proportion of the sample that is captured on the filter obtained by the routine procedure can be calculated. The efficiency of the procedure was 67% (standard error = 2%, n = 8, and coeff. of var. = 8.4%) for our tallgrass prairie soils (silt loam and silty clay loam). Procedure efficiency was 90.5% (standard error = 1.4%, n = 8, and coeff. of var. = 4.3%) for a potting mix containing 3 parts tallgrass prairie soils, 1 part calcined clay, and 1 part sand. The efficiency procedure should be carried out for each soil type being studied.

2. To accommodate a given soil, centrifuge speeds and times may be varied. Glycerol-to-water ratios could also be varied, but we have had less success with this. However, we may have been playing with the glycerol concentration at times when other factors were more critical.

3. From our experience, it appears that obtaining the proper dilution of the soil sample may be the most critical factor. If too many hyphae are present, they all clump together and are lost to the pellet that is routinely tossed following the second (75 × g) centrifugation. In addition, too many hyphae makes counting intersects difficult, time-consuming, or even impossible (when hyphae are tangled together, accurate numbers of intersects cannot be distinguished). We found in testing a range of dilutions for two different soil types that the length of hyphae per gram of soil increased as the amount of soil extracted decreased, but that a point was reached where hyphal lengths became independent of soil dilution. It is likely that this point varies depending on soil type, and it may also vary somewhat with hyphal densities.

4. The sonication step will lyse cells making determinations of viability impossible. For our purposes, having total lengths were more important than knowing viability, and sonication freed more hyphae from soil aggregates. In sandy or other soils, sonication may not be necessary; however, sonication was also found to effectively knock hyphae from roots. If maintaining viability is important, alternatives to sonication could include brief blending or simply vigorous shaking after soaking in water or more dilute sodium hexametaphosphate (to reduce osmotic stress). However, these measures may or may not knock hyphae off the roots. Also, if sonication or blending energy is too strong or long, hyphae may be broken into segments that are too short to capture with the nylon mesh filter or to score accurately.

Quantification of External Hyphae

View slides at 160× magnification. Using the gridline intersect method, count intersections for 6 horizontal and 6 vertical lines (alternating lines seen in the 10×10 eyepiece grid reticle). Do this for 70 fields of view (~10% of filter surface area) by moving the stage randomly back and forth in a zig-zag fashion across the entire filter (takes practice!). Mycorrhizal hyphae stain blue, are aseptate, have a characteristic "knobby" appearance, and dichotomous branching (see Nicholson, 1959). To control error, the number of counts per field of view should average at least 2, and an average count of 3 to 5 per field of view gives very good results. If counts routinely exceed 20-30 per field of view (usually due to clumps of entangled hyphae), consider reducing amount of soil or increasing dilution.

Calculations

Note: The following demonstrates calculations using dimensions for our Leitz Ortholux II at 160×, reticle, and filter funnels. Specific dimensions must be determined for each microscope and filtration setup.

1. For our reticle at 160×:

   Total line length = 0.595 mm per line × 12 lines = 7.14 mm.
   Area covered by reticle = 0.354 mm^2.

   Thus, from Newman (1966): $R = \pi An/2H$ or, $R = n[\pi A/2H]$,

   where R = hyphal length per reticle area, A = reticle area, n = number of intersects, and H = total line length.

   For our reticle this translates to: $R = n \times 0.07788$.

2. To determine hyphal length for the entire filter (R'), given that the filterable area of the membrane = 213.8 mm^2:

   $R' = R \times [213.8\ mm^2/0.354\ mm^2] = R \times 604$.

3. Calculate sample dry wt (SDW) by multiplying original moist sample weight (~5 g) by dry wt/wet wt ratio. Then calculate the actual dry wt of the extracted soil (ADW):

   $$\text{ADW} = \text{SDW}/302.5 \text{ mL} \times 6 \text{ mL}/287 \text{ mL} \times 20 \text{ mL}.$$

   The 302.5 mL comes from the 300 mL of liquid added to 5 g moist soil plus an assumed average volume for the soil of 2.5 mL (Schmidt and Paul, 1982; Wollum, 1982). The 287 mL is the 6 mL aliquot plus the 281 mL of diluent. These numbers may have to be adjusted if (1) the soil weight or aliquot volumes are changed or (2) the soil density is unusual.

4. Finally, hyphal length (in meters) per g dry soil in our system

   $$= \bar{n} \times 0.07788 \times 604/\text{ADW}/1000/C_E,$$

   where $\bar{n}$ = mean number of intersects from 70 fields of view and C_E is the correction for the efficiency of the procedure (see Note 1 for the extraction procedure).

Recent efforts to develop image analysis methods for scoring the membranes appear to indicate that the grid line intersect method may underestimate hyphal lengths. We believe this may be due to only scoring intersections on every other line of the reticle. We may be missing significant numbers of hyphae that are shorter than the distances between the lines being scored. The equation for calculating hyphal length per reticle area (Newman, 1966) is based on cutting root segments to the appropriate length for the size of the grid. Obviously, the lengths of hyphal segments cannot be controlled in the same way that root segment lengths are. Therefore, it may be better to score intersects with all lines of the reticle (22 instead of 12). We have not yet tested this out against the results from image analysis to see if the lengths are more comparable. If all lines of the reticle are scored, then the average number of counts per field of view will increase and it may be possible to score fewer fields of view. Of course, this will depend on hyphal densities and the evenness of hyphal distributions across the membrane, which may be evaluated by standard statistical procedures. If 22 lines are scored, then the calculations presented above must be modified as indicated below.

Total line length = 0.595 mm per line × 22 lines = 13.09 mm, and $R = n \times 0.04248$.

Therefore, hyphal length (in meters) per g dry soil in our system

$$= \bar{n} \times 0.04248 \times 604/\text{ADW}/1000/C_E.$$

Materials

Reagents

1. Sodium hexametaphosphate solution: 35.7 g/liter (final volume)
2. 50% glycerol: vol./vol. with d.i. water
3. Cargille Type A non-drying immersion oil (low fluorescence and low viscosity) for microscopy
4. Trypan blue stain: 1:2:2 lactic acid:glycerol:DI H_2O by volume, with 0.6 g trypan blue per liter

Equipment

1. Sixteen 600-mL beakers (two for each 5-g soil sample)
2. Eight 25-mm sintered glass Millipore-type filter holders with 15-mL funnel capacity
3. Eight 125-mL heavy-wall sidearm filter flasks
4. Eight 10-mL wide-mouth pipettes (purchased glass ones work best but they can be made by cutting off the tips of disposable pipettes), plus one 5-mL and two 10-mL pipettes
5. One 50-mL graduate cylinder
6. One 250-mL volumetric flask and one 1-L volumetric flask
7. Glass stirring rod
8. Glass slides with frosted ends or labels, and 24×30 mm cover slips
9. Extra beakers to hold reagents in use
10. Sieve with 2-mm openings (No. 10)
11. Metal spatula
12. Weigh boats
13. Eight 50-mL transparent hard plastic (PMP) long-conical centrifuge tubes (Nalge 3100) and eight 15-mL translucent soft plastic (PP) conical centrifuge tubes with caps (Corning 25319)
14. Filters with 20-µm mesh openings cut to 25-mm diameter from filter fabric [Nitex nylon (Cat. No. 3-20/14) from Tetko Inc., Elmsford, NY]. These can be reused many times.
15. Plain white cellulose nitrate filters (25-mm diameter) with 1.0 to 1.2-µm pore size [Micro Filtration Systems, Dublin, CA (Cat. No. A120A025A) or Whatman WCN type (Cat. No. 7190 002)].
16. Lab wash bottle
17. Fine-tipped forceps
18. Vacuum pump and manifold set up to accommodate 8 filter flasks
19. Magnetic stir plate and 1.5-inch stirring bar
20. Vortex mixer
21. Top-loading balance accurate to 0.01 g
22. Sonicator
23. Centrifuge equipped with 8-place swinging bucket head
24. Compound microscope with a 1-cm 10×10 grid reticle

References

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